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Article (Submitted Version)

Brissett, Nigel C, Martin, Maria J, Pitcher, Robert S, Bianchi, Julie, Juarez, Raquel, Green, Andrew J, Fox, Gavin C, Blanco, Luis and Doherty, Aidan J (2011) Structure of a preternary complex involving a prokaryotic NHEJ DNA polymerase. *Molecular Cell*, 41 (2). pp. 221-231. ISSN 1097-2765

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Structure of a Pre-Ternary Complex Involving a Prokaryotic NHEJ polymerase

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Running title: NHEJ Polymerase pre-ternary complex formation

SUMMARY

In many prokaryotes, a specific DNA primase/polymerase (PolDom) is required for non-homologous end-joining repair of DNA double-strand breaks. Here, we report the crystal structure of a catalytically active conformation of *Mycobacterium tuberculosis* PolDom, consisting of a polymerase bound to a DNA end with a 3' overhang, two metal ions and an incoming nucleotide but, significantly, lacking a primer strand. This structure represents a unique example of a polymerase:DNA complex in a pre-ternary intermediate state. This polymerase intermediate occurs in solution, stabilizing the enzyme on DNA ends and promoting nucleotide extension off short incoming termini. We also demonstrate that an invariant Arg²²⁰, contained in a conserved loop (Loop 2), plays an essential role in catalysis by regulating binding of a second metal ion at the active site. We propose that this NHEJ intermediate facilitates extension reactions involving critically short or non-complementary DNA ends thus promoting break repair and minimizing sequence loss during DSB repair.

Summary: 153 words. Manuscript: 45,138 Characters.

INTRODUCTION

Two major cellular pathways have evolved to repair DSBs: non-homologous end-joining (NHEJ) and homologous recombination (HR) (Daley et al., 2005; Mahaney et al., 2009). In HR, an intact duplex, homologous to the break site, acts as an alternative copy that templates for the re-synthesis of the broken strands (Helleday et al., 2007). In contrast, NHEJ does not require extensive homology but instead directly joins DNA ends together in a template-independent manner. Consequently, NHEJ can be utilised by non-cycling cells, when the absence of a homologous donor precludes DSB repair by HR and, for this reason, it has become the major pathway for repairing DSBs in mammalian cells (Helleday et al., 2007).

The higher eukaryotic NHEJ complex consists of Ku, DNA-PKcs and the ligase IV:XRCC4:XLF (LXX) complex (Daley et al., 2005; Mahaney et al., 2009). Upon formation of a DNA break in cells, Ku and DNA-PKcs binds with high affinity to the ends of DSBs, maintaining the termini in close proximity. Subsequently, Ku recruits the LXX complex, thereby directing ligase IV activity. A host of other proteins have also been implicated in eukaryotic NHEJ, including DNA polymerases λ and μ (PolX family), the nuclease Artemis and PNKP (polynucleotide kinase and phosphatase). These additional factors are involved in the replacement, removal or modification of damaged or incompatible DNA termini to produce ligatable DNA ends.

More recently, a functionally homologous NHEJ repair apparatus has been characterised in prokaryotes (Weller et al., 2002; Della et al., 2004; Pitcher et al., 2007a). The mycobacterial NHEJ complex, composed of Ku and a multifunctional DNA ligase D (LigD), can recognise, process and reseal DSBs (Della et al., 2004; Pitcher et al., 2005; Pitcher et al., 2007b). This primordial NHEJ complex is present in many bacterial species, suggesting that this repair pathway is physiologically important for genome stability in prokaryotes (Bowater and

Doherty, 2006). NHEJ-deficient stationary phase cells (*Mycobacteria*) and spores (*Bacillus*) are sensitive to agents that produce DSBs (Weller et al., 2002; Moellar et al., 2007; Pitcher et al., 2007c), indicating that NHEJ is also predominantly utilised by non-replicating prokaryotic cells.

The mycobacterial LigD, in addition to its ligase domain, also contains polymerase (PolDom) and nuclease domains (NucDom), which can process DSBs prior to ligation (Della et al., 2004). The prokaryotic NHEJ polymerases (PolDom) are members of the Archaeo-Eukaryotic Primase (AEP) superfamily (Aravind and Koonin, 2001; Weller and Doherty, 2001; Iyer et al., 2005). Historically, primases have been regarded as specialised polymerases with roles restricted to the synthesis of RNA primers at replication forks (Frick and Richardson, 2001). However, this limited role for AEPs in DNA metabolism has been challenged by the discovery of a wide variety of structurally homologous bacterial AEPs (Pitcher et al., 2007a) that possess multiple polymerase activities, including gap-filling activities (Della et al., 2004; Zhu and Shuman, 2005; Zhu et al., 2006; Pitcher et al., 2007b). In addition to these activities, a reduced template-dependence and flexible active site enables these enzymes to generate template distortions, primer realignment and lesion bypass, which greatly enhances their roles in DSB repair (Pitcher et al., 2007b).

Uniquely among the domains that constitute *Mt-LigD*, PolDom physically interacts with Ku (Pitcher et al., 2005), suggesting that LigD recruitment to DSBs is accomplished primarily via this interaction. Ku also appears to enhance polymerase extension activity (Pitcher et al., 2005). PolDom can also facilitate the association (synapsis) of non-complementary DNA ends, thus promoting end-resection prior to gap-filling and ligation (Brissett et al., 2007). Here, we provide structural and functional evidence for the existence of a catalytically competent NHEJ polymerase:DNA pre-ternary complex involved in the repair of DNA double-strand breaks.

The crystal structure of the *Mt*-PolDom in complex with dsDNA, containing a 3' overhang, reveals how these polymerases achieve templated binding of an incoming nucleotide (UTP) in the active site, in the presence of divalent metal ions, and the localised structural transitions associated with this step. Biochemical and biophysical data are presented that support the existence of this catalytic intermediate in solution. The critical role of an invariant arginine residue (Arg²²⁰), located in a conserved loop (Loop 2), in regulating metal binding and catalysis is also reported. Formation of a pre-ternary complex enhances both the binding of the polymerases to DNA ends and subsequent nucleotide extension off short incoming termini. We propose that the pre-ternary polymerase complex represents a functional NHEJ intermediate that facilitates joining of short non-complementary DNA ends and, consequently, minimizes sequence loss during DSB repair.

RESULTS

Structure of a PolDom:DNA:UTP Pre-Ternary Complex

Here, we present the crystal structure of *Mt*-PolDom in complex with dsDNA (with an 8 base 3' overhang and a recessed 5'-P) and bound incoming nucleotide (UTP) and manganese ions located in the active site. This *Mt*-PolDom complex (PolDom:UTP:Mn²⁺:DNA) represents an incomplete ternary complex, lacking a primer strand, and is therefore ascribed as a pre-ternary complex (Figure 1). The overall complex in the asymmetric unit consists of two distinct pre-ternary complexes and specific details of the DNA and nucleotide interactions with both monomers are described in the supplementary results (Figures S1 and S2).

The PolDom structure comprises amino acid residues 6-291, with no electron density observed for nine residues at the C-terminus (PDB ID:3PKY). PolDom interacts with the recessed 5'-phosphate of the downstream strand (D) (Figures 1 and S1) and orients the 3'-overhanging end of the templating strand (T), which is splayed out by ~105° (Figure 1B). Only two bases from the 3'-overhanging strand have observable density in the refined structure, suggesting that the remaining six bases are highly mobile. A single UTP is bound in the polymerase active site, which forms a Watson-Crick base-pair with a templating adenine base (A6) with two metals ions also bound in close proximity (Figure 1C). The 3'-OH group of the ribose moiety of UTP is hydrogen bonded to the amino nitrogen of Ile²³⁸. The 2'-OH group is hydrogen bonded to the side-chain hydroxyl and backbone carboxyl oxygen of Thr²³⁶, with a further hydrogen bond formed between the δN of His¹¹¹ and the 2'-OH. Gln²³⁰ interacts with the oxygen (O4') on the ribose moiety (Figure 1C). To our knowledge, this is the only reported structure of a polymerase-DNA complex containing a templated incoming base in the absence of a primer strand, a scenario that is most relevant for NHEJ polymerases. The biochemical and structural significance of this complex is described below.

Structural Evidence Supporting a Pre-Catalytic Ternary Complex

A comparison of the active sites of the binary complex (PolDom bound to GTP and metal; Pitcher et al., 2007b) with the pre-ternary complex revealed significant differences in the positioning of the nucleotides in the active sites. Notably, the position of the triphosphate tail appears to dictate if a second metal ion, absent in the binary complex, can bind in the active site (Figure 2A). The orientation of this tail is strongly influenced by template:nucleotide pairing. In the pre-ternary complex, the orientation of the templating adenine base (A6), forming a Watson-Crick base pair with the uridine base, is determined by a close stacking interaction with Phe⁶⁴ (Figure 1C). Significantly, the incoming nucleotide base in the GTP binary complex structure (with no templating base available) directly stacks against this phenylalanine (Figure 2A), changing its overall location relative to that observed in the pre-ternary complex. Thus, Phe⁶⁴ (invariant in NHEJ AEPs) plays a pivotal role in the sequential recognition and orientation of both the templating and incoming bases (Pitcher et al., 2007b) and therefore, also has an important role in determining the enzyme's affinity for a second active site metal ion.

Further support for the catalytic competency of the pre-ternary polymerase complex is provided by comparisons of the conformations of UTP (pre-ternary structure) with the Pol λ ternary complex (dUpnpp, PDB ID: 2PFO; Garcia-Diaz et al, 2007), which revealed a pair-wise atom alignment of 0.25Å, suggesting that UTP (in the pre-ternary complex) is in a catalytically competent conformation (Figure 2B). The lack of an incoming priming strand means that phosphotransfer is unable to occur and therefore, the current PolDom structure must be defined as a pre-ternary catalytic complex. However, the conformation of residues and cofactors (UTP and metals) in the active site suggests that the PolDom complex is optimally poised for extension upon engagement with an incoming primer strand.

Movement of Loop 2 in the Pre-Ternary Complex

We next examined if any major structural rearrangements had occurred in the PolDom:DNA complex upon templated binding of UTP and metal ions in the active site. Notably, the polymerase in the pre-ternary complex adopts a similar overall conformation to that observed in the apo, nucleotide- and synaptic PolDom structures (Brissett et al., 2007; Pitcher et al., 2007b), with only minor local conformational changes (see supplemental results for details). The root mean square deviation (RMSD) values are all below 1.0 Å, suggesting no major global conformational differences between the various polymerase structures. However, despite these overall similarities, a closer examination of the structural elements within the pre-ternary PolDom complex revealed that a specific conserved loop (loop 2; residues 213-223) showed large RMSD values compared to the equivalent region in other PolDom structures (*apo*, GTP, dGTP and synaptic complex). Loop 2 exists as a 3_{10} helix in all of the previously determined structures. However, this helix unravels and adopts a random coil conformation in the pre-ternary complex (Figure 2C). This significant conformational change in loop 2 results in C α position shifts of ~ 6 Å, inducing repositioning of specific side-chains on the loop. A comparison of the amino acid sequences of PolDom's revealed that loop 2 is conserved across the entire family of NHEJ primases (Figure 2D), but not the replicative orthologues, suggesting that this structural element could have a role in NHEJ. Closer inspection of the sequence of loop 2 identified an invariant arginine residue (Arg²²⁰). Notably, the side-chain of Arg²²⁰ is orientated away from the active site, upon transition from the binary to the pre-ternary complex (Figure 2C), suggesting that Arg²²⁰ may play a specific role in catalysis. The functional significance of the movement of Loop 2 is described below.

Repositioning of Loop 2 Regulates Metal Binding in the Polymerase Active Site

Does this major reorientation of Loop 2 away from the active site, upon formation of the pre-ternary complex, play any functional role in the catalytic cycle of these NHEJ polymerases? Figures 3A and 3B show views of the active site of PolDom-DNA binary complex (left panels)

and the pre-ternary PolDom complex (right panels) containing an incoming UTP base-paired to a templating adenine base (A6) with two metals ions also bound. A number of critical observations suggest that the pre-ternary complex is catalytically competent and that Loop 2 plays a critical role in activating the catalytic centre. The first notable observation is that a salt-bridge formed between Asp¹³⁹ and Arg²²⁰ (Loop 2), observed in the DNA binary complex (Figure 3A, left), is disrupted in the pre-ternary complex (Figure 3A, right). This allows a rotation of the arginine side-chain ($\sim 180^\circ$) away from the active site, enabling Asp¹³⁹ to adopt a conformation where it can favorably act as a ligand for the second catalytic metal ion (A), not present in the catalytically inactive structures (Pitcher et al., 2007b). Accordingly, mutation of this arginine to alanine (R220A) significantly reduced PolDom's ability to incorporate nucleotides (Figure 3C) but, significantly, did not alter its affinity for gapped-DNA substrates (data not shown). It appears that binding of this second metal ion triggers this conformational change and this significant rearrangement at the active site implies that Arg²²⁰ may play a direct role in catalysis.

Based on both these structural and biochemical observations, we propose that Watson-Crick base pairing of an incoming nucleotide (forming a chelating site for metal B), complementary to the templating base nearest to the recessive 5'P, acts as the primary conformational switch that induces the active site to become catalytically competent. Thus, after selection of the correct nucleotide, a second metal (A) is recruited to the active site, perhaps initially in a non-competent location requiring the interaction between Arg²²⁰ and Asp¹³⁹ to provide the necessary space/charge to bind this second metal ion. Subsequently, a metal-driven conformational change of loop 2 leads to the breakage of the Arg²²⁰-Asp¹³⁹ salt bridge, enabling Asp¹³⁹ to act as a chelating metal-binding ligand to promote repositioning of metal A into a catalytically competent location within the active site. Therefore, the capacity to form a salt bridge between Arg²²⁰ and Asp¹³⁹ (abolished in the R220A mutant), which keeps the active site in a “stand-by”

mode, is an important requisite step preceding catalysis. This pre-activated complex, loaded with a complementary nucleotide, can occur at a single DNA end before a second 3'-protruding end provides an attacking primer for nucleotide insertion and NHEJ.

Formation of a Stable Pre-Ternary Complex in Solution

To support the structural evidence for a pre-ternary polymerase complex, we measured the formation and stability of PolDom pre-ternary complexes in solution, adopting established fluorescent methodologies used to analyse polymerases ternary complexes (Kumar et al., 2008). Formation of a pre-ternary complex was assessed by adding UTP to a preformed fluorescent complex of *Mt*-PolDom:DNA:Mn²⁺, in which the 3'-protruding DNA end contains a 2-aminopurine [2AP] templating base. Titration of the UTP concentration enabled the determination of a binding constant (K_d) for nucleotide in the pre-ternary complex (*Mt*-PolDom:DNA:UTP:Mn²⁺). We observed that a stable pre-ternary PolDom complex was formed in solution, as pairing of UTP to the templating base quenched the intrinsic fluorescent signal of 2AP. Figure 4A shows the normalised fluorescence of the preformed *Mt*-PolDom:DNA:Mn²⁺ complex with varying concentrations of UTP and the ensuing quenching that accompanies base-pairing of 2-aminopurine with UTP. From these data, we calculated a K_d of 0.646μM for the binding of UTP to 2-aminopurine, determined by fitting relative quenching values to a ligand binding equation with a non-specific binding factor. The sub-micromolar range K_d for UTP binding closely correlates with the binding constants determined for nucleotide binding for other DNA polymerase ternary complexes (Kumar et al., 2008).

Next, we evaluated the formation and stability of both binary and pre-ternary DNA complexes in solution, by electro-mobility shift assays (EMSAs) using a T/D DNA molecule with a short 3' protrusion (GTT-3'), in the presence/absence of activating divalent metal ions and incoming nucleotides. As shown in Figure 4B, *Mt*-PolDom produced a stable binary complex with DNA

in the absence of metal ions, observed as a sharp retarded band. Addition of either 10 μM Mn^{+2} or 100 μM Mg^{+2} did not significantly affect the formation and stability of such a binary complex. However, addition of both activating metal ions and CTP (the required nucleotide to form a correct pre-ternary complex), significantly enhanced the stability of the complex formed. Conversely, addition of non-complementary nucleotides had either an inhibitory effect (GTP; see Figure 4B) or a negligible effect (ATP; data not shown) on the stability of the polymerase pre-ternary complex.

Functional Significance of the Pre-Ternary Polymerase Complex

Although *Mt*-PolDom can form a stable pre-ternary complex in solution, is this complex catalytically active? Moreover, is such a preformed complex beneficial for catalysis? To address these questions, we tested if formation of a pre-ternary complex, prior to the encounter with a primer, resulted in altered primer extension activity. For this, we used a trinucleotide to minimize terminal transferase-like nucleotide additions driven by *Mt*-PolDom. A schematic of this experiment is shown in Figure 5A. Figure 5B shows that the amount of 3G primer extended (dG-dG-dG-CMP) is significantly higher (~30% more efficient) when PolDom was pre-incubated with the templating molecule (..GCC-3'), correct nucleotide (CTP) and metal ions (MnCl_2) in the absence of primer, than when all of the reactants were added simultaneously. This beneficial effect of the pre-incubation did not occur when other combinations of co-factors were pre-incubated (Figure 5B). Therefore, the only situation in which a catalytic advantage was observed (see the reaction kinetics in Figure 5C) was after addition of the four components needed to form a pre-ternary complex. Similar results were also obtained with primers of different lengths and matching different DNA ends (not shown).

Pre-Ternary Complex Formation Enhances Extension off Short Incoming Termini

The results described above support the catalytic significance of a pre-ternary complex in

solution and suggest that such complexes enable these enzymes to extend off otherwise unstable termini (e.g. very short or blunt termini) or to deploy additional extension activities (e.g. terminal transferase). In the pre-ternary complex, the position of a number of conserved positively charged residues (e.g. Lys¹⁷⁵ & Arg²⁴⁶), which line the active site pocket, has altered significantly with respect to the PolDom:DNA binary complex, modulating the shape and charge of the ridge (Figure 3B). This conformational change enlarges the cavity, most likely to facilitate acceptance of an incoming primer strand (Figure 3B, right panel), in agreement with the catalytic advantage provided by a pre-formed pre-ternary complex.

Replicative AEP primases are able to bind a “primer” as small as a single NTP, allowing the subsequent synthesis of a dinucleotide at the initiation step of RNA primer synthesis (Figure 6A). As bacterial NHEJ polymerases are closely related to these canonical AEPs, we tested the minimal length of DNA (incoming end) that can be accepted as a “primer” by *Mt*-PolDom (both DNA binary and pre-ternary complexes) to determine if these enzymes have a similar capacity to accept and extend off very short “primers”, which in the context of NHEJ are short DSB overhangs. As shown in Figure 6B, three different molecules were tested as potential primers to be extended with CTP (the nucleotide pre-loaded in a pre-ternary complex formed at a 3' protruding (CCG) DNA end). The scheme on the left shows the degree of complementarity of the three primers used (3G, 2G or G). Primer extension (p + CMP) was observed with 3G and 2G primers, but not with GTP, and was mostly dependent on the presence of the DNA end (pre-ternary complex). Figure 6C shows that the minimal primer (2G) could only be extended when the DNA termini was complementary (CCG), and not with a non-complementary end (TTG). Moreover, a complementary NTP (a requisite to form and stabilize a pre-ternary complex) was required to form an extended product (dG-dG-CMP) (Figure 6C). Similar results were also obtained with primers matching a different DNA end (not shown). Together, these results establish that formation of a pre-ternary polymerase complex significantly enhances the

extension off short incoming termini (Figure 6B and C).

Discussion

Conventional replicative and lesion bypass DNA polymerases extend off dsDNA substrates, containing both primer and template strands, in a 5' to 3' direction. In contrast, polymerases involved in DSB repair must be capable of binding to and extending off non-canonical DNA polymerase substrates, including 3' over-hanging termini lacking a primer strand. Recent studies on the bacterial NHEJ polymerases have revealed some of the unusual activities associated with these repair enzymes that enable DNA extension under the most extreme conditions (Pitcher et al., 2007a). For example, a homodimeric arrangement of the mycobacterial NHEJ polymerases can facilitate the association of two incompatible 3'-protruding DNA ends, via microhomology-mediated synapsis, forming a stable end-joining intermediate (Brissett et al., 2007). This synaptic complex reflects an intermediate bridging stage of the NHEJ process, prior to end-processing and ligation. In this way, the polymerase restores the continuity of the ds DNA helix, catalyzing a conventional 5'-3' extension reaction occurring on one DNA end, but templated in trans by a second (synapsed) DNA end.

Although recent studies have provided unique insights into polymerase-mediated orchestration of break synapsis, the order of substrate binding events and mechanism by which these NHEJ polymerases catalyse end-extension is still poorly understood. To address this question, we have elucidated the crystal structure of a pre-ternary intermediate of *Mt*-PolDom bound to DNA and NTP and shown that this complex is relevant for specific DSB repair processing events. This catalytically competent complex consists of a *Mt*-PolDom monomer, containing two metal ions and a templated nucleotide (UTP) in its active site, bound to a dsDNA end with a 3' overhang but, significantly, lacking a primer strand (Figure 1). To our knowledge, this structure represents a unique example of a polymerase-DNA complex captured in a pre-ternary intermediate state, relevant for NHEJ.

Is the pre-ternary complex, described here, physiologically relevant for prokaryotic NHEJ polymerase extension reactions? Although the pre-ternary complex lacks an incoming primer strand, which provides the attacking nucleophile (3'OH), a comparison of the positioning of the nucleotide base, phosphate tail, active site ligands and divalent metal ions to those in the active site of a ternary polymerase complex (Pol λ) provides compelling evidence that the PolDom pre-ternary complex is catalytically competent, even in the absence of the primer strand (Figure 2B). The possibility of preforming a pre-ternary complex in solution by incubating the necessary components (*Mt*-PolDom, DNA end, complementary nucleotide and activating metal ions) in the absence of a primer, allowed us to demonstrate its physiological relevance in accelerating NHEJ reactions, probably by providing a “ready to use” primer binding site. By testing the activity of the pre-ternary PolDom complex with different ssDNA primers, we concluded that the minimal primer utilizable by these enzymes is a dinucleotide, as *Mt*-PolDom was not proficient at polymerizing off a single nucleotide “primer” (Figure 6A). The fact that *Mt*-PolDom cannot efficiently use a single nucleotide as a primer indicates that, although PolDom is evolutionarily related to replicative AEPs, its physiological activity as a primase has effectively been lost and, instead, these polymerases have evolved to have a more restricted capacity to bind short incoming DNA termini, enabling them to perform more specialized roles in NHEJ break repair processes.

From a mechanistic point of view, this study has identified a second conserved loop (loop 2), which plays a prominent role in activation/inactivation of the catalytic centre. The conformation of loop 2 changes significantly upon the templated-binding of the correct incoming nucleotide, which induces the rotation of the Arg²²⁰ side-chain ($\sim 180^\circ$) away from the active site in the pre-ternary complex (Figure 3A). Mutation of this invariant residue abolished the extension activity (Figure 3C) but, significantly, did not alter enzyme binding to other DNA substrates, such as gapped DNA. A comparison of the structures of the PolDom-DNA binary

versus the pre-ternary complex reveals the sequential movements that occur in the active site, induced by the binding of both a templating base and an incoming nucleotide (Figure 3A). The invariant active site residue Phe⁶⁴, which stacks against the base of the incoming nucleotide in the PolDom:GTP binary complex (Figure 2A), now stacks against the base of the templating nucleotide (A6) in both PolDom:DNA and pre-ternary complexes, orienting this base and also maintaining (together with Phe⁶³) the major kink in the template strand ($\sim 105^\circ$). In replicative DNA polymerases, aromatic residues are employed as an essential part of a fidelity mechanism that scrutinizes pairing of the correct incoming base with the templating base, thus acting as a molecular gatekeeper to limit the incorporation of incorrect/mismatched bases during elongation (Johnson et al., 2003). We propose that an analogous fidelity mechanism involving the two invariant phenylalanine residues also occurs in the bacterial NHEJ polymerases, but in the absence of the primer strand, thus ensuring that the correctly templated incoming base is bound in the active site prior to the encounter with the incoming end/primer providing the attacking 3'OH.

This phenylalanine-mediated (Phe⁶⁴) stacking interaction with the templating base in the pre-ternary complex also promotes the movement of the incoming nucleotide (UTP) into the active site and, together with the loss of specific contacts (e.g. Arg²⁴⁶, Lys¹⁷⁵, Lys⁵²) promotes the correct repositioning of the α -phosphate group of the incoming nucleotide for catalysis (Figure 2A). This re-orientated α -phosphate moiety, together with Asp¹³⁹, forms a second metal binding site (A) not present in the binary structure, which is required for the two metal catalytic mechanism common to all DNA polymerases (Brautigam and Steitz, 1998). The binding of the second metal, in turn, promotes breakage of the salt bridge between Arg²²⁰ and Asp¹³⁹, repositioning this aspartate into a catalytically favourable alignment with the other catalytic aspartates, the α -phosphate group and the two bound metal ions, to form an activated pre-ternary intermediate awaiting the arrival of the nucleophile (3'OH of the primer strand) (Figure

3A). The primer-independent NTP induced-fit step is schematically shown in figure 7. The catalytic incompetence of the R220A mutant highlights the importance of the interaction of Arg²²⁰ with Asp¹³⁹. We propose that the maintenance of this amino acid pairing provides a significant barrier to catalysis until the enzyme becomes optimally bound to DNA, metals, and the correct incoming templated nucleotide. Once these are bound within the active site, a sequence of structural rearrangements promotes the binding of a second metal ion (A). The affinity of Asp¹³⁹ for this second metal promotes the loss of interaction with Arg²²⁰, leading to expulsion of Loop 2 from the active site, which results in full activation of the catalytic centre. The movement of Loop 2 away from the active site, most likely, promotes this activation step in two ways. The first consequence is that breaking the salt bridge is irreversible, leading to the release of the acidic side-chain of Asp¹³⁹, which is involved in the binding of the second metal (A) within the active site, ensuring that it is optimally poised for catalysis. The second notable consequence, induced by the reorientation of Loop 2, is a significant change in the ridge (Figure 3B) that surrounds the active site, which most likely allows the 3'-OH of the incoming primer strand to bind in the active site to form the ternary complex. Further steps of catalysis, PPi release, and ligation would terminate the NHEJ process. An illustration of some of the different complexes formed during the NHEJ cycle is depicted in figure 7.

Evolutionary Implications

Since the discovery of NHEJ in prokaryotes, there has been much speculation as to why AEP primases were selected as the primary NHEJ polymerases in bacteria. Prokaryotic NHEJ polymerases evolved, from primordial AEPs with an innate ability to make short RNA primers, into a distinct clade of adaptable end-joining nucleotidyltransferases capable of performing multifunctional roles required to process non homologous DNA ends during DSB repair. By comparing the structures and sequences of PolDom's with those of canonical replicative primases (e.g. *Pfu* primase; Pitcher et al., 2007b), it is clear that both share a common primase

catalytic core but there are several distinctive functional adaptations. These differences include the existence of loop insertions (e.g. loops 1 and 2) and a positively charged phosphate-binding region in PolDom, that distinguish the NHEJ AEPs as a distinct primase family (Figure S3 and S4). We have shown that the formation of a non-primed pre-ternary complex with the NTP pre-loaded is beneficial, as it facilitates catalysis to occur as soon as the incoming primer is recruited to form a ternary complex. This mechanism is reminiscent of the initiation reaction carried out by replicative primases (Figure 6A), in which the order of events is very similar: firstly, a binary complex between enzyme and DNA is formed, followed by a pre-ternary complex in which the 3' nucleotide is initially stabilized, followed by recruitment of the 5' nucleotide (the "primer"), to form a ternary complex. Thus, as a reflection of their common phylogenetic origins, both NHEJ AEPs and replicative AEPs can catalyze an unorthodox addition of a ribonucleotide in the 3'-5' direction, followed by (in the case of AEPs) conventional (5'-3') elongation events. The innate ability of AEPs to accept short primers may have influenced evolutionary selection of these enzymes by prokaryotes to become the NHEJ polymerase. Indeed, many bacteria encode additional AEP orthologues whose physiological roles remain to be determined.

In eukaryotes, PolX family members were adopted as the NHEJ polymerase of choice and the AEPs appear to be used predominantly in DNA replication processes. Is pre-ternary complex formation also relevant for eukaryotic NHEJ polymerases? It has been demonstrated that human Polμ can catalyze NHEJ extensions on very short and incompatible DNA ends (Nick McElhinny et al, 2005; Davis et al 2008), a reaction that can take advantage of a limited terminal transferase activity (Andrade et al, 2009), and that can occur with both dNTPs and NTPs. It is likely that formation of a Polμ pre-ternary complex, as that described here, triggered by the strong recognition of a 5'-recessive phosphate and a reinforced avidity for the incoming nucleotide (both properties also intrinsic to Polμ) would be beneficial to carry out non-

complementary NHEJ of minimally processed ends in eukaryotes, although this remains to be proven.

Concluding Remarks

Although a multi-protein NHEJ complex, including Ku and LigD, appears to be the major determinant for the gross physical association of DSBs (macro-synapsis), it is likely that processing enzymes (including polymerases and nucleases) within these complexes also play significant roles in orchestrating the association of the extreme termini of breaks (micro-synapsis), especially non-homologous ends, thus co-ordinating the processing of these termini prior to ligation. Although the structures of several PolDom-DNA complexes have provided “snap-shots” of intermediates in the catalytic cycle of the bacterial NHEJ polymerases, the challenge now is to obtain structures of ternary complexes in the act of extending off the 3'OH terminus of an incoming primer strand.

Experimental Procedures

Crystallographic Analysis of the Pre-Ternary PolDom Complex

Crystallization, diffraction data collection and refinement of the *Mt*-PolDom:DNA:UTP complex was performed as described in the supplementary section.

DNA Substrates

PAGE-purified oligonucleotides were 5' end-labelled with [γ -³²P]ATP by polynucleotide kinase. The oligonucleotides used to generate the DNA substrates were the following: for gapped substrates, P15 (5'-TCTGTGCAGGTTCTT-3'), T32 (5'-TGAAGTCCCTCTCGACGAAGAACCTGCACAGA-3') and 5'phosphate-containing D16 (5'-GTCGAGAGGGACTTCA-3'); for NHEJ template/downstream (T/D) substrates, oligonucleotide 5'-CCCTCCCTCCCGCC-3' (GCC3') was used as template, hybridized to a downstream oligonucleotide 5'-GGGAGGGAGGG-3' (DP), which contains a phosphate at the 5'-end. Oligonucleotides 5'-GGG-3' and 5'-GG-3' were used as primers. For EMSA, template oligonucleotide 5'-CCCTCCCTCCCGTT-3' (GTT3') was hybridized to DP to form a T/D molecule.

Construction and Purification of *Mt*-PolDom Mutant Proteins

Site-directed mutagenesis (QuickChange, Stratagene) was performed on the over-expression plasmid for *Mt*-PolDom, DNA constructs were sequenced and transformed into *E. coli* B834(DE3)pLysS. Wild-type and mutant *Mt*-PolDom variants were over-expressed and purified as described (Pitcher et al., 2005).

Amino Acid Sequence Comparisons

A multiple alignment of different primases and primase domains from members of the AEP family was done using the program MULTALIN (<http://prodes.toulouse.inra.fr/multalin/>).

EMSA and Polymerization Assays

Assays were carried out essentially as described (Pitcher et al., 2007b). EMSAs were employed to analyse the interaction of *Mt*-PolDom with NHEJ intermediates in a final volume of 12.5 μ l, containing 50 mM Tris-HCl (pH 7.5), 0.1 mg/ml of BSA, 1 mM DTT, 4% glycerol, 5 nM labelled DNA and different concentrations of *Mt*-PolDom. After incubation for 10 min at 30 °C samples were mixed with 3 μ l of 30% glycerol and resolved by native gel electrophoresis on a 4% polyacrylamide gel (80:1 (w/w) acrylamide/bisacrylamide). For standard (gap-filling) polymerization assays, the incubation mixture (20 μ l) contained 50 mM Tris-HCl (pH 7.5), 1 mM MnCl₂, 1 mM DTT, 4% glycerol, 0.1 mg/ml of BSA, 5 nM gapped DNA, the indicated concentration of NTPs and either wild-type *Mt*-PolDom or mutant R220A. After 30 min of incubation at 30 °C, reactions were stopped by adding loading buffer (10 mM EDTA, 95% (v/v) formamide, 0.03% (w/v) bromophenol blue, 0.3% (w/v) cyanol blue) and subjected to electrophoresis in 8 M urea-containing 20% polyacrylamide sequencing gels. After electrophoresis, the unextended and extended DNA primers were detected by autoradiography. NHEJ polymerization assays were carried out essentially as described above, but using independent DNA template molecules (unlabelled) and short homopolymeric oligonucleotides as a labelled primer, and specific reaction times, as indicated in the corresponding figure legends.

Steady-State Fluorescence Emission Assay

Emission fluorescence data for 2-aminopurine (2AP) DNAs (ATD-Bio) and *Mt*-PolDom complexes were collected using a Varian Cary Eclipse fluorescence spectrophotometer. Samples were excited at 360 nm and fluorescence emission data scans collected between 340-470nm. Band pass slits were 10 and 20 nm for excitation and emission channels, respectively. Solutions containing 200 nM 2AP-labelled DNA, 200 nM *Mt*-PolDom, 50 mM Tris (pH 7.5),

150 mM NaCl, 10 mM MnCl₂ and 4% glycerol were titrated with stock solutions of UTP. Intrinsic protein fluorescence was subtracted. Fluorescence values were plotted and relative quenching was fitted to the equation, $F = ([UTP] / K_d [UTP]) + N_s [UTP]$. F is the relative quenching specific to the complex and N_s is the non-specific binding constant.

SUPPLEMENTAL DATA

Supplementary data including results, four figures and one table can be found at <http://www.cell.com/molecular-cell/supplemental/S.....>

ACKNOWLEDGEMENTS

We declare that none of the authors have a financial interest related to this work. AJD laboratory supported by grants from BBSRC and MRC. LB laboratory supported by grants from Ministerio de Ciencia y Tecnología (BFU2006-14390/BMC and CSD2007-00015) and Comunidad de Madrid (P2006/BIO306), and by an institutional grant to Centro de Biología Molecular “Severo Ochoa” from Fundación Ramón Areces. MJM is a recipient of a contract from the Comunidad de Madrid. We acknowledge the ESRF for synchrotron radiation facilities and thank the beamline staff of ID16.

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Figure Legends

Figure 1. Crystal Structure of the Pre-Ternary Complex of *Mt*-PolDom:DNA:UTP

(A) Ribbon representation of the pre-ternary complex depicting the interaction of PolDom (blue) with the dsDNA and UTP. Loops 1 and 2 are highlighted in salmon pink showing their spatial relationship to the bound DNA and UTP molecules. DNA is coloured green for downstream (D) strand and red for templating (T) strand. The bound UTP molecule is coloured cyan and the catalytic manganese ions are pink.

(B) Schematic representation of *Mt*-PolDom binding to DNA and UTP. The unstructured 3'-overhanging DNA is shown as a faded colour as it is unstructured. DNA chains follow the same colour scheme as in (A). PolDom is represented by a blue crescent. The arrow indicates a kink angle (105°) between A6 and C5 of the template strand.

(C) A close-up view of the active site of *Mt*-PolDom highlighting the important stacking of A6 against Phe⁶⁴ and the specific interactions of PolDom with UTP and UTP with DNA (A6). The DNA and protein monomer are coloured as in (A). Hydrogen bonds are shown in grey.

Figure 2. Structural Evidence Supporting a Pre-Catalytic Ternary Complex

(A) Schematic view of the changes affecting nucleotide positioning, comparing the binary (*Mt*-PolDom:GTP; PDB ID: 2IRX) complex (lilac) versus the pre-ternary complex (blue). Metal ions (A and B) are indicated as pink spheres. Rotation of Phe⁶⁴ to establish alternative interactions either with the incoming nucleotide (GTP) or with the templating base (A6) is highlighted.

(B) UTP is in a catalytically competent conformation in the pre-ternary complex. Superposition of the catalytic site of Polλ (PDB ID: 2PFO; Garcia-Diaz et al, 2007) onto the catalytic site of the PolDom pre-ternary complex. Catalytic aspartates from *Mt*-PolDom are coloured dark blue with the UTP and templating base from the same structure coloured light blue. Catalytic aspartates from the Polλ structure are coloured red and the DNA, 2'-deoxyuridine 5'-alpha, beta-imido-triphosphate (dUPnPP) coloured salmon pink. Mn²⁺ ions are coloured pink and Mg²⁺ ions are coloured green.

(C) Ribbon representation highlighting the conformational changes associated with loop 2, largely affecting the orientation of Lys²¹⁷ and Arg²²⁰. *Mt*-PolDom structures corresponding to

the binary (PDB ID: 2IRX) and pre-ternary complexes were superposed. The two metal ions present in the pre-ternary complex are indicated in deep pink. The region corresponding to loop 2, and residue Arg²²⁰, are indicated either in red (binary) or in pink (pre-ternary). Loop 1 is also depicted (yellow) to show the relative orientation of these structural elements.

(D) Amino acid sequence alignment of the PolDom region encompassing loop 2 in the two main groups of bacterial LigD enzymes (see Figure S3 for enzyme nomenclature, amino acid positions and a more extended alignment). Invariant or highly conserved amino acids are indicated in red and orange, respectively. Dots above the *Mt*-PolDom sequence indicate the catalytically relevant Arg²²⁰ (studied in this paper) and the metal ligand carboxylate residue Asp²²⁷. Below the alignment is the consensus sequence, represented as a sequence logo (Crooks et al. 2004), showing that Arg²²⁰ is the most conserved residue in loop 2.

Figure 3. Role of Loop 2 in Regulating the Active Site of PolDom

(A) Schematic view of the conformational changes affecting loop 2 (green) and some selected residues acting as metal or nucleotide ligands, comparing the *Mt*-PolDom:DNA binary complex (PDB ID: 2R9L; left) versus the pre-ternary complex (right). Metal ions (A and B) are indicated as pink spheres. Incoming nucleotide (UTP) is coloured cyan. The templating nucleotide (A₆) present in the pre-ternary complex, is indicated in red.

(B) Changes in electrostatic surface potential in the active site pocket, comparing the *Mt*-PolDom:DNA binary complex (PDB ID: 2R9L; left) versus the pre-ternary complex (right). Loop 2 residues (Lys²¹⁷, Arg²²⁰) are highlighted, as well as the residues involved in the formation of the positively charged ridge (in blue) above the active site (Lys⁵², Lys¹⁷⁵, Arg²⁴⁴, Arg²⁴⁶).

(C) Gap-filling activity of *Mt*-PolDom loop 2 mutant, R220A. DNA synthesis reactions on a 5'-P-containing (yellow) 1nt-gapped substrate (5 nM) were carried out as described previously (Pitcher et al. 2007a), in the presence of either wild-type or mutant (R220A) *Mt*-PolDom (400 nM), 1 mM Mn₂Cl₂, and 100 μM of either the correct (ATP) or incorrect NTP. After incubation for 30 min at 30°C, extension of the 5'-labelled primer strand was analysed by 8 M urea-PAGE (20% polyacrylamide gel) and autoradiography.

Figure 4. Formation of a Stable Pre-Ternary Polymerase Complex in Solution

(A) Titration of *Mt*-PolDom:2AP-labelled DNA complex with free UTP in the presence of MnCl_2 . The main graph shows the normalised fluorescence of *Mt*-PolDom:2AP-labelled DNA complex (200 nM *Mt*-PolDom, 200 nM ddC terminated 2-aminopurine labelled DNA, 50 mM Tris pH 7.5, 150 mM NaCl, 10 mM MnCl_2 , 4% glycerol) titrated with varying concentrations of UTP. Inset, graph showing the relative fluorescence quenching as a function of UTP concentration (◆) overlaid with data fit to the non-linear regression ligand binding equation (—).

(B) A stable pre-ternary complex formed in solution. As detected by electrophoretic mobility shift assays (EMSA), *Mt*-PolDom binds 3'-protruding (...GTT3') T/D ends with a 5'-P to form a PolDom:DNA binary complex (arrow). Addition of activating metal ions and a complementary nucleotide (CTP) allows formation of a more stable pre-ternary complex. Conversely, a stable pre-ternary complex is not formed in the presence of a non-complementary (GTP) nucleotide. EMSA assays were performed with 600 nM *Mt*-PolDom and 5 nM DNA, as described (Pitcher et al., 2007). When indicated, cofactors (MgCl_2 100 μM ; MnCl_2 , 10 μM and nucleotides (either CTP or GTP 100 μM) were added.

Figure 5. Formation of a Pre-Ternary Complex Enhances Nucleotide Extension off an Incoming Primer Strand.

(A) Schematic representation of the experimental steps reported in parts (B) and (C) (below). Some of the components needed for the extension reaction (*Mt*-PolDom (grey crescent), a template molecule (blue) with a 5'-P (yellow dot) and a GCC-3' protrusion, dGdGdG (red) as primer, CTP and manganese ions) were allowed to interact during a pre-incubation step, as indicated in B for each case. Subsequently, the remaining components required for the reaction to occur were added, and the extension step can proceed.

(B) NHEJ polymerase extension reactions, following the scheme described in (A). Concentrations used were: 600 nM *Mt*-PolDom, 1mM MnCl_2 , 100 μM CTP, 5 nM labelled primer, cold CCG3' and 25 nM template DNA. During pre-incubation for 30 min at 4°C, the components present are indicated by a + sign, while missing components are indicated by a – sign. At the start of the reaction period, added components are indicated by a black + sign, while components already present are indicated by a grey + sign. Reactions were performed for 30 min at 30°C, the samples were run on a 6M Urea-20% polyacrylamide gel and autoradiographed.

(C) Kinetics of NHEJ polymerase extension reactions following the scheme described in (A). – Pre-inc indicates pre-incubation of *Mt*-PolDom only; + Pre-inc indicates pre-incubation of the components needed to form the pre-ternary complex (*Mt*-PolDom, nucleotide, template DNA, Mn^{2+}). Insertion reactions were stopped at the times indicated, samples loaded on a 6M Urea-20% polyacrylamide gel and autoradiographed.

Figure 6. Minimal Length of DNA that can be Accepted as a “Primer” by the Pre-Ternary PolDom Complex

(A) A comparison of the reaction steps performed by replicative AEPs versus NHEJ AEPs. See text for details.

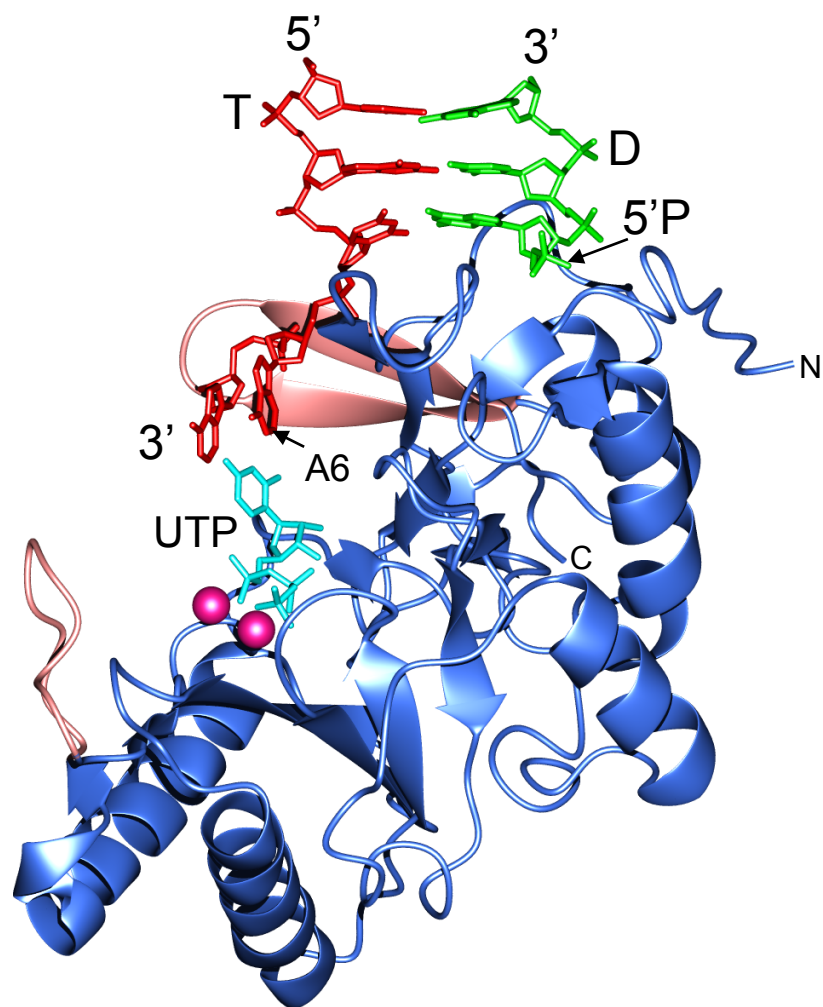
(B) A 3'-protruding (GCC-3') DNA molecule (DNA1; 25 nM), with a 5'P (yellow sphere) was used as the DNA end to form a pre-ternary complex in the presence of 600 nM *Mt*-PolDom, 1 mM $MnCl_2$ and 100 μ M CTP. Different DNA molecules (dGdGdG, dGdG or dG; indicated in red), labelled at the 5'-end, were tested as primer strands (5 nM) to accept the CTP substrate pre-loaded in the pre-ternary complex. After incubation for 24 h at 4°C, primer extension products (p+CTP) were separated by 8M Urea-30% PAGE and detected by autoradiography.

(C) As in (B), but using two different DNA molecules (DNA1 and DNA2), both capable of forming a pre-ternary complex with CTP, and different NTPs (100 μ M) as indicated. The minimal primer (dGdG) could only be extended with CTP when a complementary template (DNA1) was present but not with an uncomplementary template (DNA2)

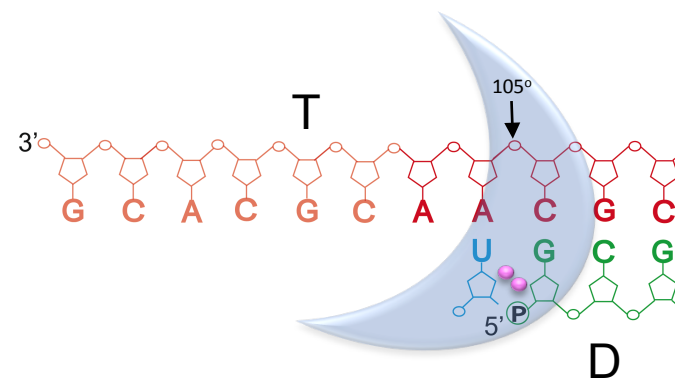
Figure 7. Catalytic Cycle of a Prokaryotic NHEJ Polymerase

Initially, a binary complex between the PolDom enzyme (grey crescent) and DNA (T/D; blue) is formed, mainly stabilized via interactions with the 5'P. Binding of an incoming NTP (green) forms a pre-ternary complex, still incompetent for catalysis as it lacks metal A. Upon template selection and relocation of the complementary NTP and the two metals, A and B, at the correct site (representing a primer-independent NTP induced-fit step) a pre-ternary pre-catalytic complex is formed. Such an activated complex, as that described here, is ready for DNA end-joining, allowing the 3'-OH of the incoming primer strand (red) to bind in the active site to form the ternary complex. Further steps of extension, PPi release, dissociation, and ligation (performed by the ligase domain of LigD), complete the DNA repair process.

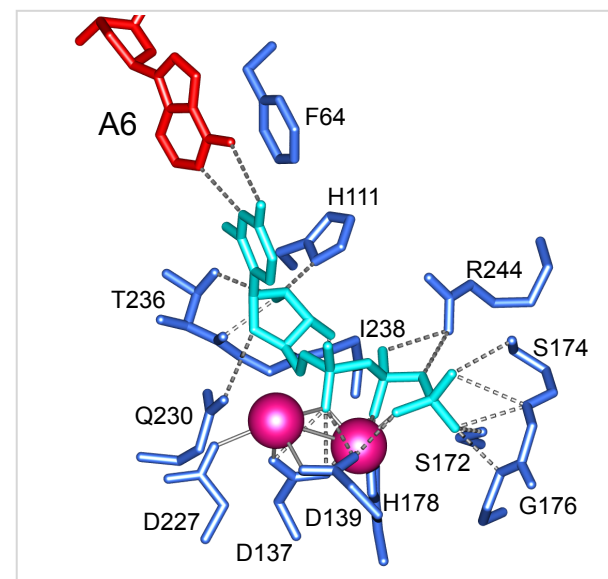
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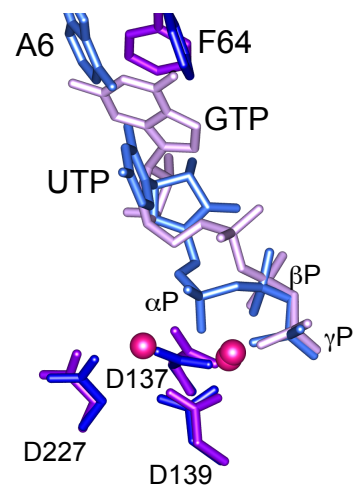
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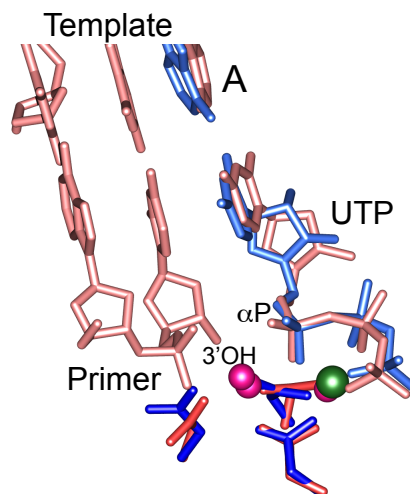
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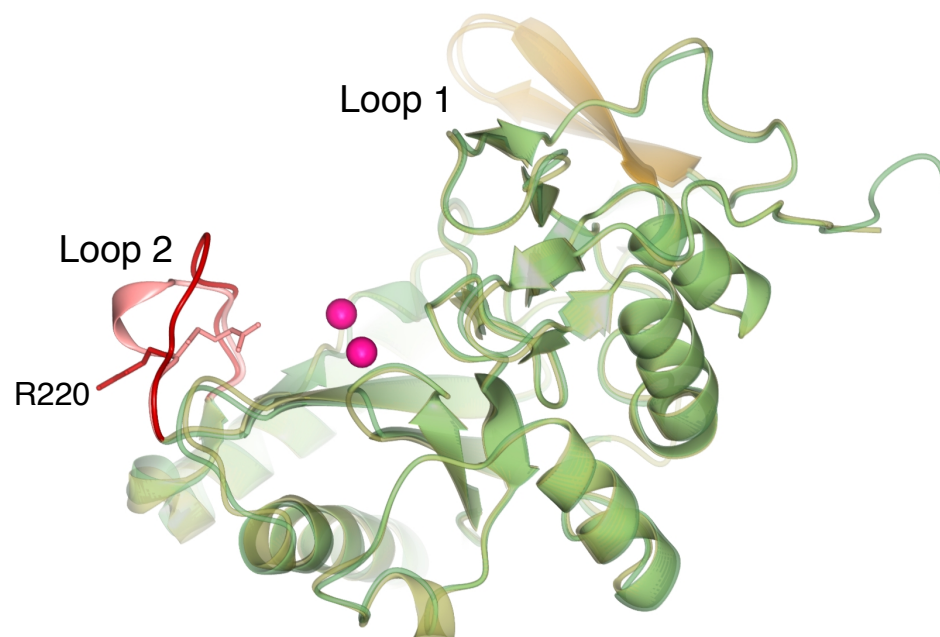
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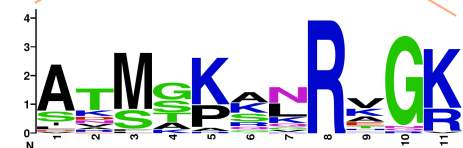
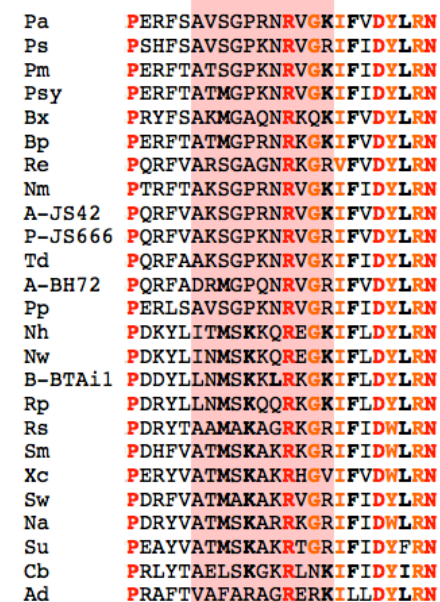
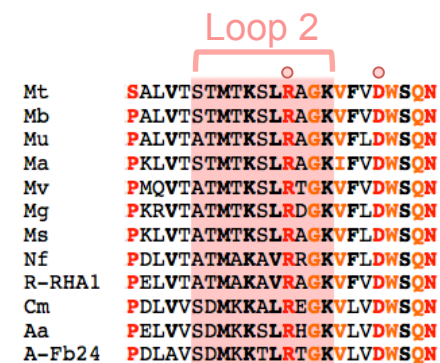
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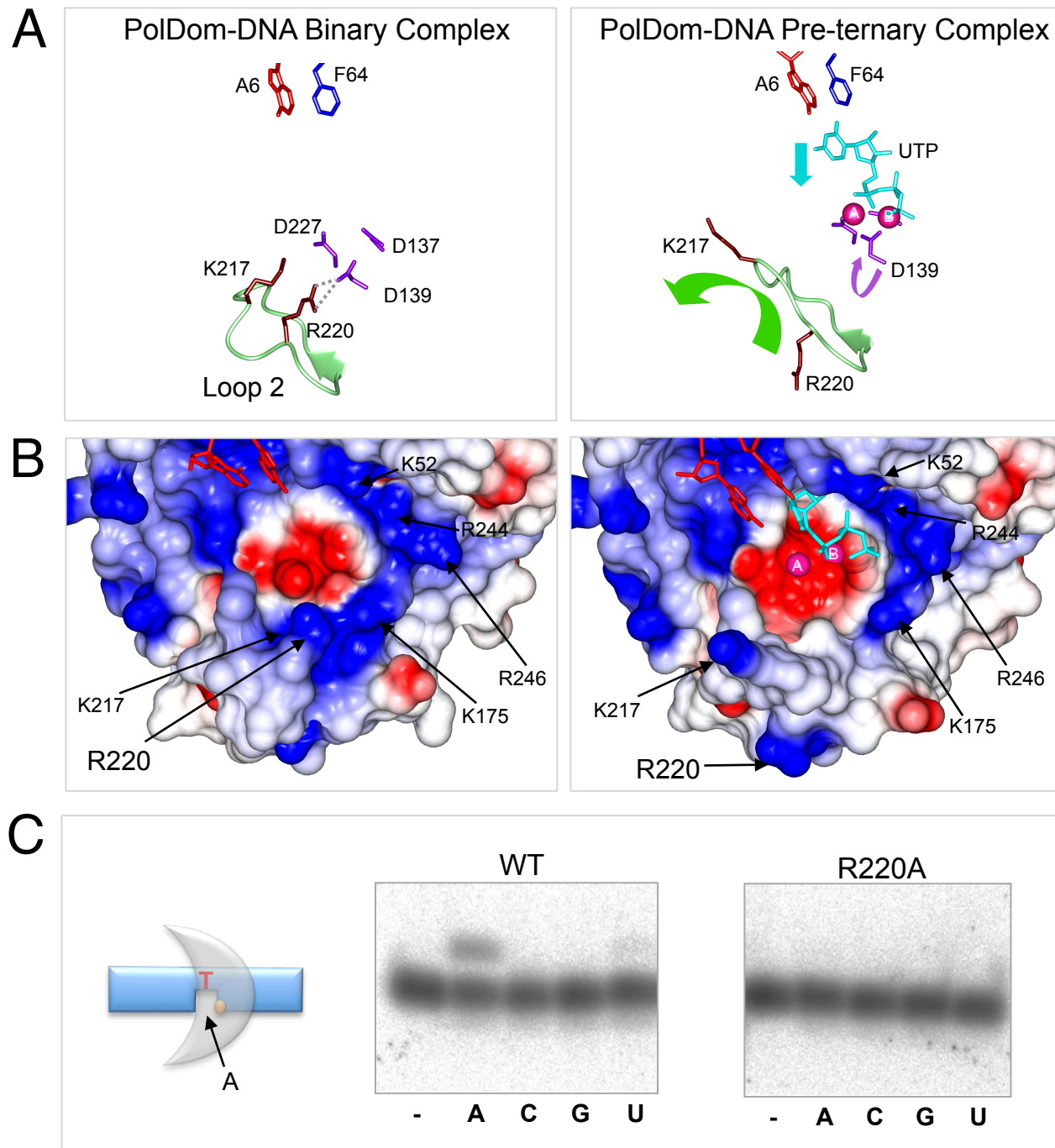


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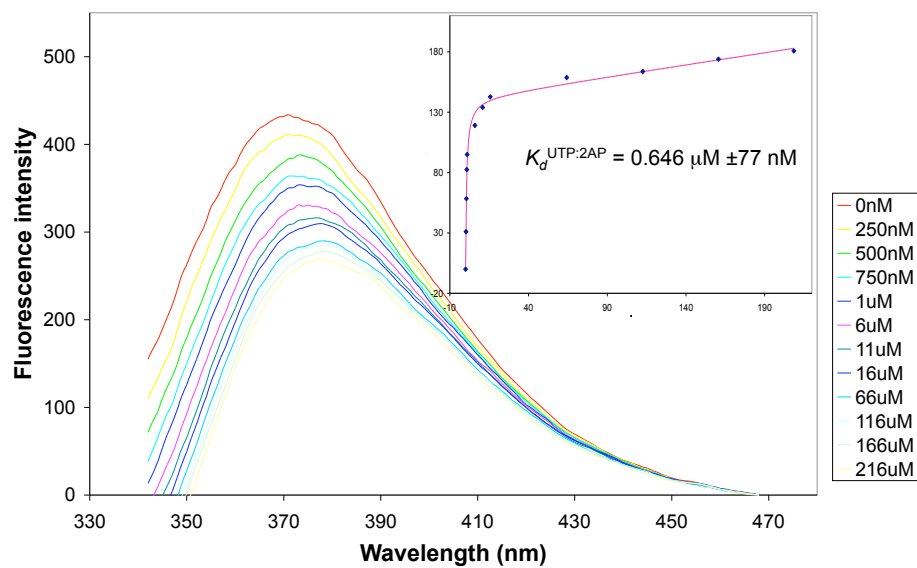
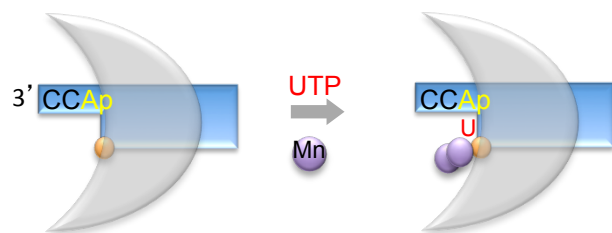


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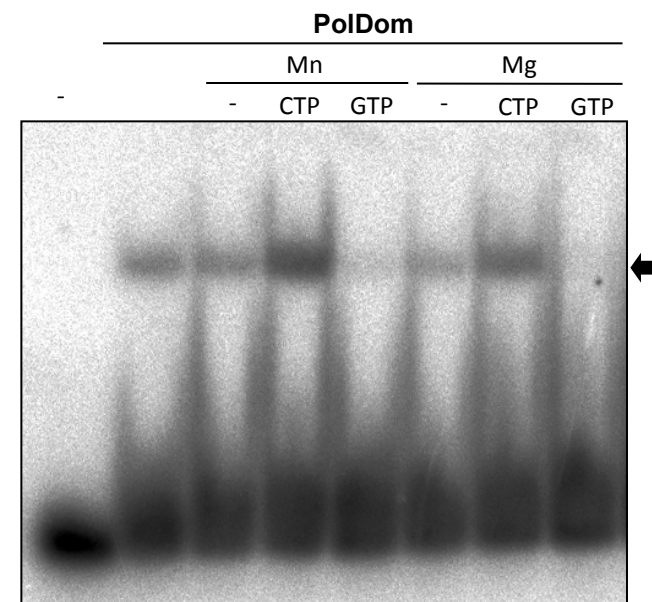
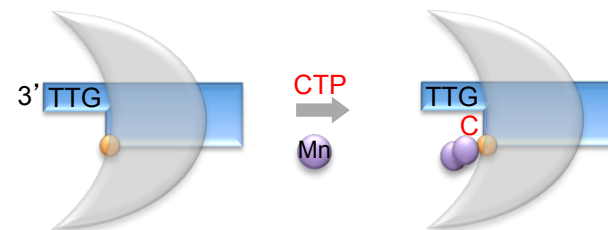


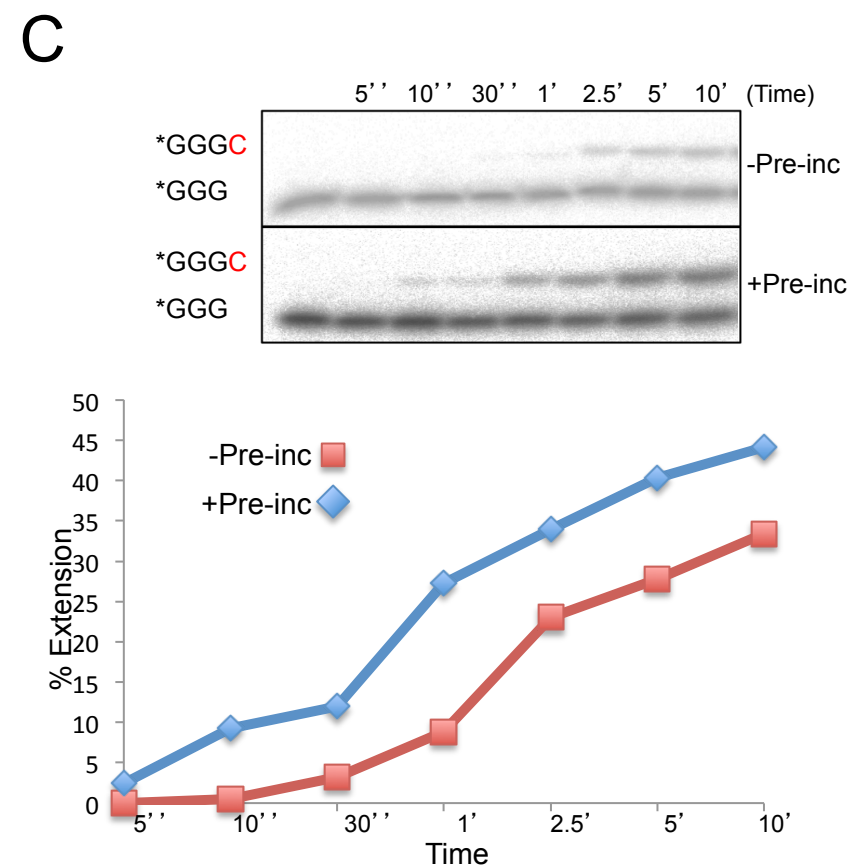
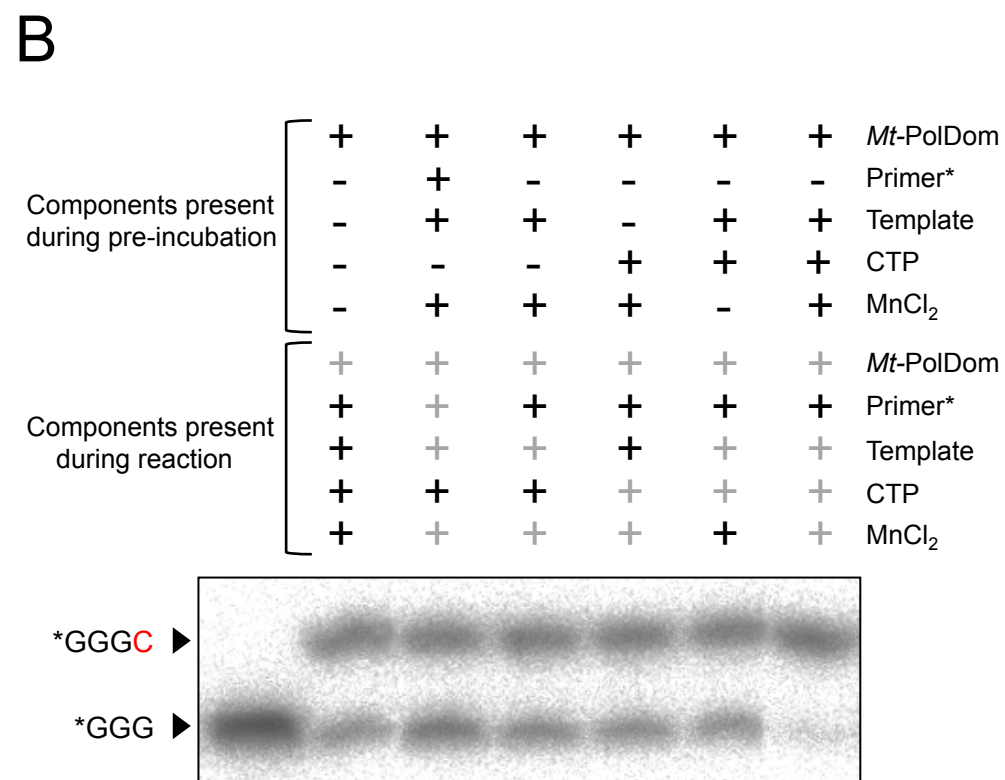
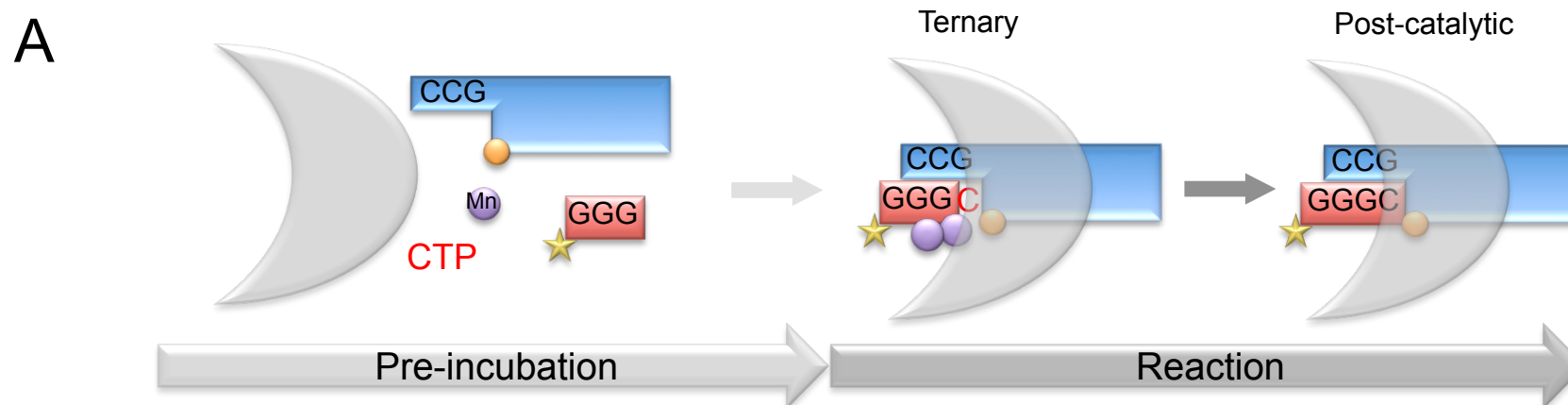


A DNA binary complex Pre-ternary complex



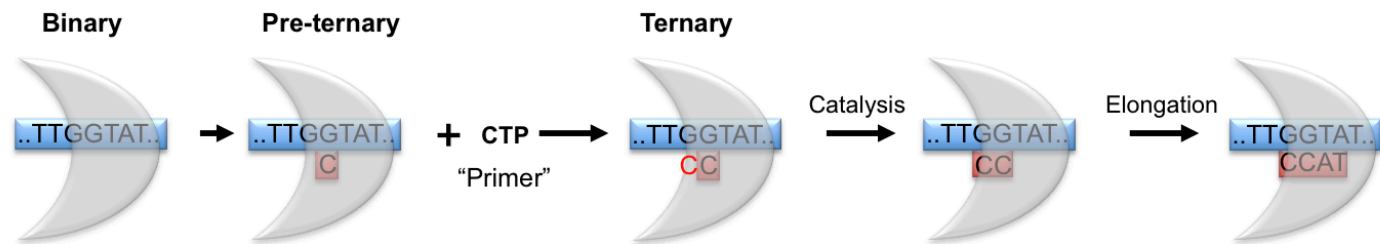
B DNA binary complex Pre-ternary complex



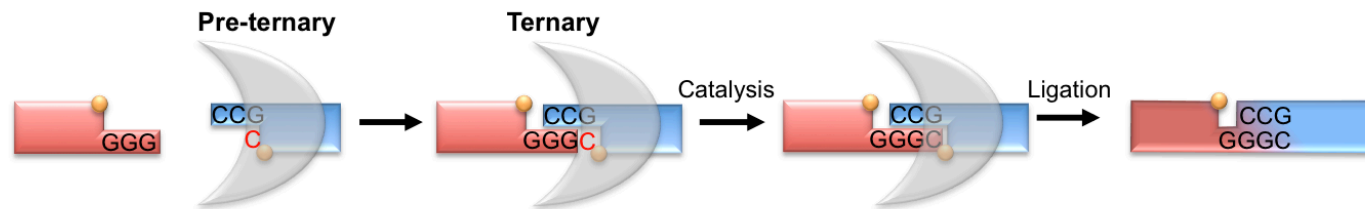


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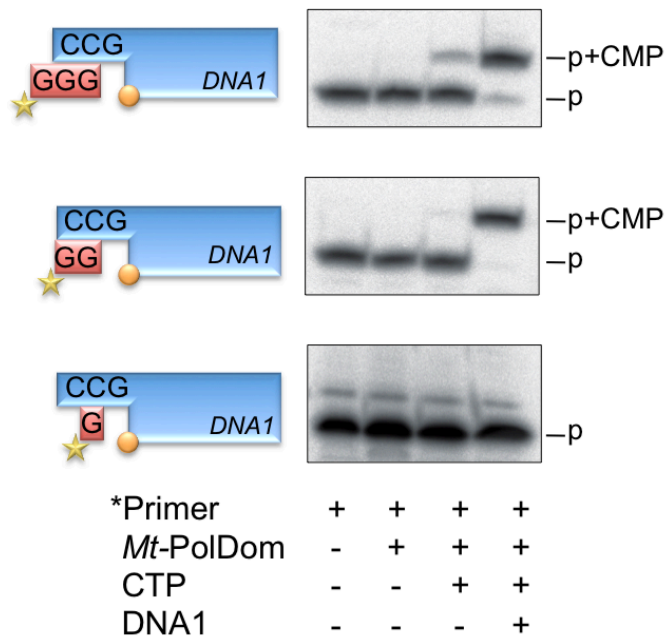
Replicative AEPs



NHEJ AEPs



B



C

